

RbcL* Sequences Provide Phylogenetic Insights among Sister Species of the Fern Genus *Polypodium

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ABSTRACT.—Opinions on the phylogeny and classification of the Polypodiaceae sensu stricto have been particularly divergent and recent DNA sequencing analyses have supported the hypothesis that this group should be allied to the higher indusiate ferns. Within the genus *Polypodium*, isozymic and restriction site analyses demonstrated that interspecific genetic identities are quite low, suggesting that sequences of the conservative *rbcL* gene would be appropriate for addressing open systematic questions. Average infrageneric sequence divergence (1.87%) was sufficient to generate reliable species differences, and the skewness test of randomly generated trees suggested that the data were non random and probably phylogenetically informative. A strict consensus of the eight most parsimonious trees supported the monophyly of the *P. vulgare* complex, and decay analysis showed that primary tree branches were well supported. Most of the phylogenetically informative nucleotides were at third codon positions. Neotropical *Polypodium* species were chosen a priori as a close out-group, and emerged as a sister group to the *P. vulgare* complex. *Polypodium amorphum* + *P. appalachianum* formed a clade that was morphologically supported by the synapomorphic feature of sporangiasters in the sori. Although the indication that *P. glycyrrhiza* was related to the Asian species *P. fauriei* was not unexpected, the close association of these two species with *P. scolieri* was quite surprising, and provided insights on the possible origin of the latter species through adaptation to stressful habitats. Whereas the long held hypothesis of a close association between *P. australe* and *P. macaronesicum* was upheld, the *rbcL* sequence data could provide no insights on the phylogeny of the enigmatic Hawaiian endemic, *P. pellucidum*. *Pleopeltis thyssanolepis*, until recently classified as a *Polypodium*, was solidly allied to *Pleopeltis macrocarpa* var. *complanata*. It is anticipated that future molecular analyses will provide additional insights on the evolutionary history of the Polypodiaceae.

Reconstructing the evolutionary history of ferns has been a continuing challenge to systematists, and has been complicated by persistent questions about the interpretation of morphological characters. Not only are ferns structurally less complex than seed plants, but their fossil history is quite incomplete (Lovelis, 1977). Different interpretations of the available data have resulted in divergent opinions concerning the phylogenetic reconstruction of fern lineages (cf. Ching, 1940; Christensen, 1938; Holttum, 1949; Mickel, 1974; Pichi Sermolli, 1977; Tryon and Tryon, 1982; Wagner, 1969). Each of the dissimilar hypotheses about the evolutionary history of ferns can be supported by apparently reasonable evaluations of available characters. However, most of these features are attached to leaves, and it is difficult to identify which are phylogenetically informative and which have been influenced by environmental pressures on leaves. Thus, molecular data, which can serve as an independent

test of existing phylogenetic hypotheses, are particularly welcome in systematic work with ferns.

Few families of ferns have seen more divergent treatment than the Polypodiaceae. From Christensen's (1938) view that this family encompassed nearly all of the "modern" ferns", through Ching (1940), Holttum (1949), Pichi Sermolli (1977), and Wagner (1969), who agreed that the Polypodiaceae were a discrete family that shared a common ancestor with the Gleicheniaceae, to Mickel (1974), who allied polypodiaceous genera with the Hymenophyllaceae, all authors seemed to agree that the exindusiate Polypodiaceae lineage was separate from the main radiation of the indusiate ferns. Jarrett (1980), however, examined details of sporangial structure, gametophyte form, and indument complexity, and concluded that similarities of the Polypodiaceae to more ancient groups was the result of parallel evolution. Rather than emphasizing the similarity of the vegetative features of the Polypodiaceae to those of the Gleicheniaceae, Jarrett suggested that derived reproductive features shared by the Polypodiaceae and the bulk of the indusiate ferns provided strong evidence of alliance with this group. She could not determine the origin of the exindusiate condition typical of the Polypodiaceae, but she noted that there were other exindusiate species scattered among the more typically indusiate ones. These observations take on a special significance in light of the conclusions from other recent DNA sequencing studies (Hasebe et al., 1994; Wolf et al., 1994) and the global analysis performed as part of this symposium (Hasebe et al., 1995) that clearly root members of the Polypodiaceae among the "higher" leptosporangiate ferns.

The complex centering on *Polypodium vulgare* L. includes a group of diploids and polyploids whose interrelationships have been studied extensively. For many years, this complex was considered to be a single, polymorphic, circumtemperate species. But, when Manton (1950) published the results of her cytogenetic work on the group, she reported different ploidal levels in both European and North American populations. Shivas (1961a, b) extended these important chromosomal investigations by initiating crosses between each element in the complex and then analyzing meiotic chromosome behavior in these artificial hybrids of known parentage. These experiments provided a sound genetic basis for resolving species limits and polyploid origins among Old World elements of the *P. vulgare* complex. Once the boundaries of interbreeding were recognized, subtle but stable morphological descriptors for these "biological" species could be established. Through these studies, Shivas (1961b) was able to bring great stability to the circumscription of European *Polypodium*. In the New World, Lloyd and Lang (1964) recognized three cytotypes of the single species *P. virginianum* L. in eastern North America and a total of six cytotypes assigned to four species in the west. In reviewing the relationships among the diploid taxa they indicated that (p. 168), "It is now apparent that there are two major species complexes in America, one centering around *P. virginianum* and the other about *P. glycyrrhiza*." One of the most distinctive morphological attributes defining these two complexes was the presence of paraphyses (currently called sporangiasters sensu Baayen and

Hennipman [1987]) in the sori of members of the *P. virginianum* group and the absence of these structures in the *P. glycyrrhiza* group. The combination of morphological, biogeographical, and chromosomal analyses developed by Shivas (1961a,b), Lloyd and Lang (1964), and Lang (1971) provided testable hypotheses about species circumscription and speciation processes in the *P. vulgare* complex.

Using this set of hypotheses as a starting point, Haufler et al. (1995b) surveyed isozymic variability among diploids and polyploids. These isozyme studies yielded robust tests for hypotheses of allopolyploid origins and also improved the resolution of diploid species boundaries (Haufler and Windham, 1991). However, genetic identities among diploid species were quite low (average=0.34) and, consequently, isozyme analyses of diploid phylogeny were suspect, because band homologies could not be demonstrated with certainty. Subsequent studies of restriction site variation among members of the *P. vulgare* complex (Haufler et al., 1995a) suggested that diploid members of the complex could have a common ancestor and confirmed the Lloyd and Lang (1964) hypothesis that there were two morphologically-defined sub-groupings within the *P. vulgare* complex. Yet, the observation that the sub-groups were separated by at least 28 restriction sites demonstrated that the species in the complex were remarkably distinct from each other. The large genetic distance evident from both restriction site and isozymic databases laid the foundation for considering more conservative features such as the sequence of nucleotides in the *rbcL* gene of the chloroplast.

In this study we sequenced a 1323 base region of the *rbcL* gene from diploid species representing both sub-groups of the *P. vulgare* complex, as well as additional *Polypodium* diploids whose relationship to the *P. vulgare* complex was suspected and two diploids from the closely allied genus *Pleopeltis*. Using a series of close and distant out-groups, we tested available hypotheses about the interrelationships among members of the *P. vulgare* complex and developed a set of new, testable hypotheses about related species. We also considered the interrelationships of genera in the Polypodiaceae and how these genera illuminate the phylogeny of the higher leptosporangiate ferns.

MATERIALS AND METHODS

Collection localities for the plants that supplied DNA for this study are listed in Table 1. Although most sequences were obtained from single individuals, when more than one collection was sequenced there were only minor differences that did not affect tree topologies. Other molecular studies have also discovered high genetic relatedness among infraspecific populations (Haufler et al., 1995a, b)

The selection of potential relatives as close outgroups was based primarily on overall morphological similarity with ingroup taxa. To arrive at these decisions we also consulted with a set of willing experts on tropical ferns, especially *Polypodium*. These experts included Elias de la Sota (1973 and pers. comm.), Robbin C. Moran, Alan R. Smith, Robert G. Stolze, Rolla M. Tryon,

TABLE 1. Collection localities for plants that were used to obtain *rbcL* sequences. Unless otherwise indicated, all sequences were obtained from individual plants and all voucher specimens are deposited at KANU.

<i>P. amorphum</i>	Mile marker # 25, Route 14, East of Washougal, Skamania Co., Washington. <i>Haufler and Soltis</i> s.n.
<i>P. appalachianum</i>	East cove, Eastman Lake, Grantham, about 20 mi. SE of Lebanon, Sullivan Co., New Hampshire. <i>Haufler</i> s.n.
<i>P. australe</i>	Cliffs at Anstey Cove, Torquay Coast; Natl. Grid Ref. 20/64.8/93.5, England. <i>Haufler, Jermy, and Rabe</i> s.n. Rock walls in church yard, Torbryan, W. of Ipplepen, SSW of Newton Abbot; Natl. Grid Ref. 20/82/67, England. <i>Haufler, Jermy, and Rabe</i> s.n.
<i>P. fauriei</i>	Epiphytic on trees near Nikko, Japan. <i>Yahara</i> s.n. (Voucher at TI)
<i>P. glaberulum</i>	Epiphytic on tree trunks, Km 104.8, Rte. 175, Oaxaca, Mexico. <i>Barrington and Haufler</i> 922.
<i>P. glycyrrhiza</i>	Roadside stream near Korbel, SE of Blue Lake, Humboldt Co., California. <i>Haufler and Mesler</i> s.n.
<i>P. macaronesicum</i>	Tenerife, Canary Islands. <i>Hennipman</i> FS-082. No voucher.
<i>P. pellucidum</i>	Epiphyte in rain forest at Volcano National Park, Hawaii. <i>Li, Haufler, and Werth</i> s.n.
<i>P. plesiosorum</i>	Epiphytic on trees along slopes near microwave tower, Txomtehuitz, NE of San Cristobal, Chiapas, Mexico. <i>Barrington and Haufler</i> 924. On volcanic substrate, Mpio. Jilotepec, NE of Jalapa, Vera Cruz, Mexico. <i>Barrington, Haufler, and Palacios-Rios</i> 925.
<i>P. rhodopleuron</i>	Epiphytic on tree branches, moist ravine, 8 km north of Altotonga, Mpio. Atzalan, Vera Cruz, Mexico. <i>Haufler, Barrington, and Palacios-Rios</i> 926.
<i>P. scouleri</i>	On cliffs near Point Reyes, Marin Co., California. <i>Haufler</i> s.n. On bases of redwood trees, along Glenwood Heights Rd., Arcata, Humboldt Co., California. <i>Haufler and Mesler</i> s.n.
<i>Pleopeltis thyssanolepis</i>	Epiphytic on trees along streambed, ca. 39 km N of Rte. 190, on Rte. 131, just S of km 163, Oaxaca, Mexico. <i>Andrews</i> 1000.
<i>Pleopeltis macrocarpa</i> <i>var. complanata</i>	Epiphytic along road near Pencion Flormar, Alajuela Province, Costa Rica. <i>Andrews</i> 456.

and Warren H. Wagner. In addition, following the discussion of Lellinger (1993) and Hennipman et al. (1990), there appears to be strong support for the hypothesis that *Polypodium* is a primarily New World genus with origins in tropical America. Asiatic species that had been assigned to *Polypodium* have been segregated into the related genus *Goniophlebium* (Rödl-Linder, 1990). Thus, it is consistent to consider *Polypodium* species from the American tropics as sister taxa to the temperate complex that is the main focus of the research described here. We chose three species from a cluster of presumably closely allied taxa. Two of these, *P. plesiosorum* Kunze and *P. rhodopleuron* Kunze, can be easily mistaken for each other morphologically. The third, *P. glaberulum*

Mickel and Beitel, is more distinctive. The nature of these ingroup and outgroup relationships is explored further below.

The selection of more distant outgroups was based on the global *rbcL* analysis (Hasebe et al., 1995) and on the hypotheses developed by Jarrett (1980). Thus, we chose not to use sequences of members of the Gleicheniaceae or *Dipteris* as outgroups, and instead employed sequences of *Davallia* (provided by Mitsuyasu Hasebe) and *Polystichum* (provided by Paul Wolf) in our analyses.

High-molecular-weight DNAs (nuclear, chloroplastic, and mitochondrial) were isolated from leaf tissue using a protocol modified from the mini-prep CTAB procedure of Doyle and Doyle (1987). Approximately 0.8 gm of leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. Three to four ml of CTAB-4% PVP solution at 55°C were added to the frozen powder and ground further. The remainder of the DNA isolation procedure followed Doyle and Doyle (1987). Concentration of each DNA sample was determined using a Hoefer mini-fluorometer. Tris-EDTA was added to each sample to adjust the original concentration of DNA to 10 µg/ml for use in PCR reactions.

We followed standard procedures for obtaining the DNA sequences (Bogdanovicz et al., 1993; Ranker, 1995). To produce a 1323-base double-stranded product, PCR amplification was carried out in a Perkin Elmer thermocycler using the Z1 and Z1351R primers representing the bases 1–30 and 1351–1380, respectively, of *rbcL* from *Zea mays* L (Zurawski et al., 1981). The conditions of the amplification process were: After 3 min of denaturation at 72°C, 40 cycles of the following sequence of temperature changes: 92°C for 1 min, 45°C for 1 min, 72°C for 3 min; then a final 7 min extension at 72°C. A second round of asymmetric PCR amplification (same conditions as above, but with only one of the two primers and only 20 cycles) yielded single-stranded product that we used to generate sequences. Internal primers included Zurawski primers Z1020 and Z1204R, as well as a series of primers developed by Paul Wolf for use with ferns (F673, F1201, F259R, F454R, F673R, and F940R). These primers were used with the US Biochem, Sequenase version 2.0 kit. ³⁵S-labeled dATP was incorporated into the sequences which were visualized autoradiographically. All sequences were deposited in GenBank via the Internet using BankItTM.

The computer program PAUP Version 3.1.1 (Phylogenetic Analysis Using Parsimony, Swofford, 1993) was used to analyze the sequence data. Trees were rooted using the standard outgroup rooting option of PAUP and rooting the tree at an internal node with a basal polytomy. The data set was small enough that the branch-and-bound algorithm, which guarantees identification of the shortest tree, was run on the data sets. The program provided a table of sequence divergence values from which the percent divergence could be obtained by dividing the absolute number of base changes between taxa by the total number sequenced (1323). We also used the branch-and-bound option of the program to perform 100 replicate bootstrap analyses and to place confidence intervals on monophyletic groups (Felsenstein, 1985). The heuristic op-

TABLE 2. Sequence divergence values for sequenced taxa. Numbers above diagonal are absolute numbers of nucleotide differences. Numbers below diagonal are percent differences.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Distant	1. <i>Polystichum</i>	—	102	109	104	105	104	103	101	105	107	103	108	108	119	111
outgroup	2. <i>Davallia</i>	7.7	—	72	78	70	64	66	63	68	69	65	69	72	78	67
Close	3. <i>P. glaberulum</i>	8.2	5.4	—	34	29	35	34	34	38	35	31	40	37	60	57
outgroup	4. <i>P. plesiosorum</i>	7.9	5.9	2.6	—	14	35	30	34	37	34	31	35	35	59	54
	5. <i>P. rhodopleuron</i>	7.9	5.3	22.	1.1	—	30	29	29	32	29	24	34	30	52	45
Ingroup	6. <i>P. amorphum</i>	7.9	4.8	2.6	2.6	2.3	—	5	15	19	17	16	22	20	48	41
	7. <i>P. appalachianum</i>	7.8	5.0	2.6	2.3	2.2	0.3	—	14	18	18	15	17	19	47	40
	8. <i>P. australe</i>	7.6	4.8	2.6	2.6	2.6	1.1	1.1	—	18	18	5	19	21	45	38
	9. <i>P. fauriei</i>	7.9	5.1	2.9	2.8	2.4	1.4	1.4	1.4	—	20	21	25	21	53	46
	10. <i>P. glycyrrhiza</i>	8.1	5.2	2.6	2.6	2.2	1.3	1.4	1.4	1.5	—	17	20	12	50	41
	11. <i>P. macaronesicum</i>	7.8	4.9	2.3	2.3	1.8	1.2	1.1	0.3	1.6	1.3	—	20	18	44	37
	12. <i>P. pellucidum</i>	8.2	5.2	3.0	2.6	2.6	1.7	1.3	1.4	1.9	1.5	1.5	—	22	47	40
	13. <i>P. scouleri</i>	8.2	4.4	2.8	2.6	2.3	1.5	1.4	1.6	1.6	0.9	1.4	1.7	—	48	41
<i>Pleopeltis</i>	14. <i>Pl. macrocarpa</i>	9.0	5.9	4.5	4.5	3.9	3.6	3.6	3.4	4.0	3.8	3.3	3.6	3.6	—	17
	15. <i>Pl. thyssanolepis</i>	8.4	5.1	4.3	4.1	3.4	3.1	3.0	2.9	3.5	3.1	2.8	3.0	3.1	1.3	—

tion of PAUP was used to conduct decay analyses in which consensus trees are developed that are 1, 2, and 3 steps longer than the most parsimonious (Olmstead and Palmer, 1994) was used to test the robustness of the data set. Skewness of the distribution of 10000 randomly generated trees obtained from the data set was used to assess the likelihood that the sequence data were phylogenetically informative (Hillis and Huelsenbeck, 1992).

RESULTS

The interspecific sequence divergence values are shown in Table 2. The average sequence divergence among members of *Polypodium* was 1.87%. Among the members of the *Polypodium vulgare* complex, the lowest absolute values were found between the species pairs *P. amorphum* and *P. appalachianum* and *P. macaronesicum* and *P. australe*, both of which differed by only five bases. The greatest distance among members of the complex was between *P. pellucidum* and *P. fauriei*, which differed by 25 bases. Distances between the members of the *P. vulgare* complex and the triad of tropical American *Polypodium* species that served as the close outgroup (*P. glaberulum*, *P. plesiosorum* and *P. rhodopleuron*) were generally higher than among the members of the ingroup. These distances ranged from a low value of 24 base differences between *P. rhodopleuron* and *P. macaronesicum* and a high value of 40 base differences between *P. glaberulum* and *P. pellucidum*. The close outgroup species that shared the most morphological features with members of the ingroup (*P. plesiosorum* and *P. rhodopleuron*) also showed fewer base changes than those that appeared more morphologically distinct (*P. glaberulum* and members of the genus *Pleopeltis*, including *Pleopeltis thyssanolepis* (A. Braun ex Klotzch) E.A. Andrews and Windham, which until recently had been considered a member of *Polypodium* [Windham, 1993]).

The distribution of 10,000 randomly generated trees based on the data set was significantly skewed to the left ($g_1 = -1.417133$; $p < 0.01$). This analysis

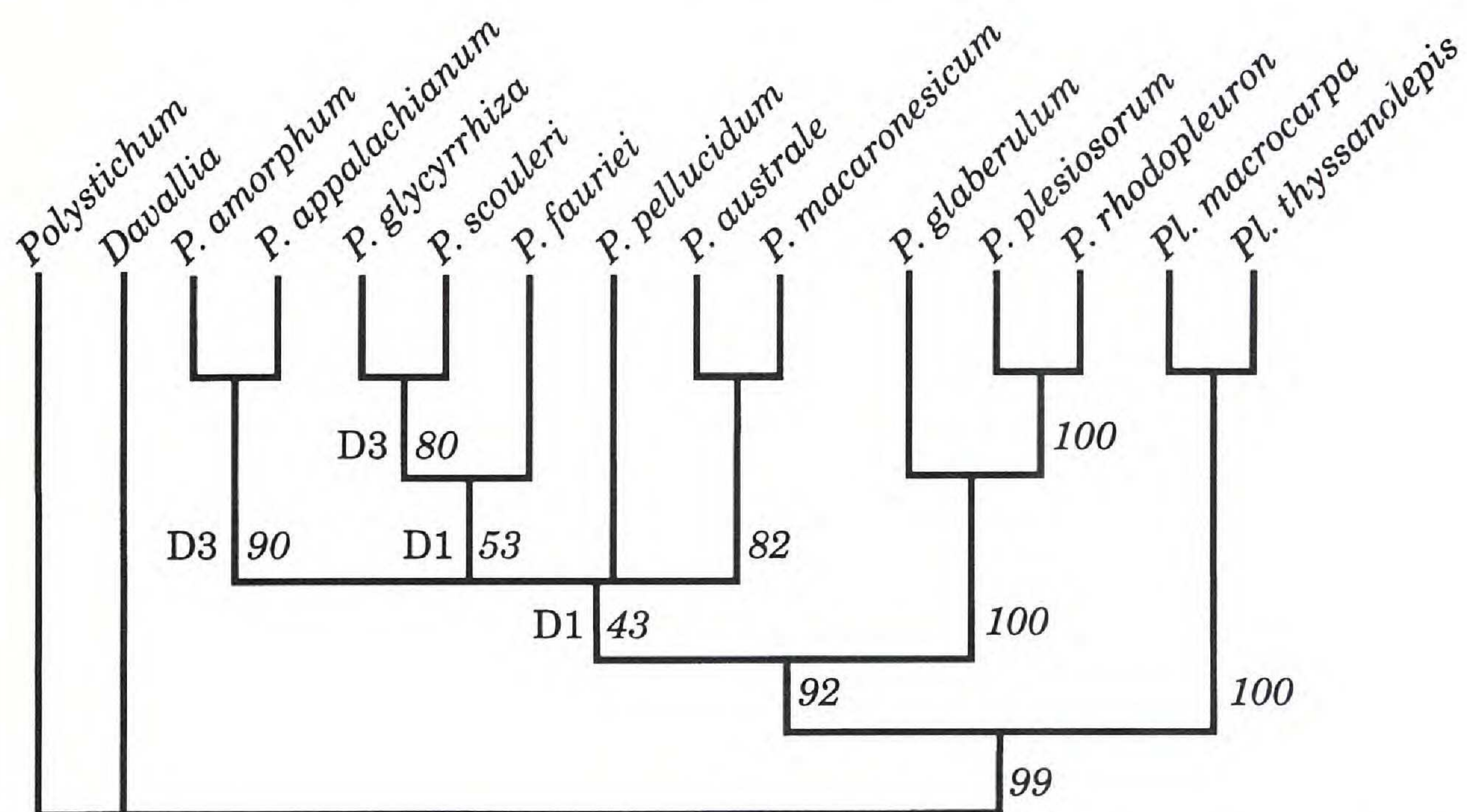


FIG. 1. Strict consensus tree of eight most parsimonious trees obtained by PAUP using the branch and bound algorithm. The tree is 286 steps long, has a CI of 0.77, and an RI of 0.61. Numbers to the right of the branches are bootstrap confidence values based on 100 replicates. D1 and D3 indicate nodes that collapsed on strict consensus trees when all trees one (D1) and three (D3) steps longer than the most parsimonious were considered. The strict consensus of all trees two steps longer than the most parsimonious did not differ from the one that was one step longer.

indicated that the data have a high probability of being phylogenetically informative (Hillis and Huelsenbeck, 1992).

Using the branch-and-bound algorithm of PAUP and including all outgroups, 8 most parsimonious trees of 286 steps with a consistency index (CI) of 0.77 and retention index (RI) of 0.61 were obtained. A strict consensus tree (Fig. 1) obtained from the eight trees supported the monophyly of the ingroup (the *P. vulgare* complex) and the intermediate position of the close outgroup of Neotropical *Polypodium* species between the more distant outgroups and the ingroup. Strict consensus trees were obtained following three steps of decay from the most parsimonious tree. At a tree length of 287, 59 trees were obtained, at 288, 259 trees were produced, and at 289, 620 trees were obtained. Two nodes collapsed at a tree length of 287 (indicated by D1 in Fig. 1), no additional nodes collapsed at a length of 288, and two more nodes collapsed at a tree length of 289 (indicated by D3 in Fig. 1). Following 100 bootstrap replicates, confidence values were assigned to each of the nodes of the original consensus tree (Fig. 1).

A series of analyses was performed that excluded information from either the first, second, or third codon position. Excluding the first codon position resulted in a tree that was identical to that obtained when all codons were included. The length of the eight most parsimonious trees dropped to 260 steps, the CI was still 0.80, and the RI remained 0.61. When the second codon position was excluded, except for the loss of resolution of *P. fauriei*, the con-

sensus tree was identical to the original tree. The length of the 16 most parsimonious trees was 270, the CI remained at 0.80, and the RI was 0.61. The strict consensus tree obtained when the third codon position was excluded clearly demonstrated that most of the information content in this analysis involved variation in this nucleotide position. The only groups that were resolved were the two *Pleopeltis* species that retained sister taxon relationship and the two most distant outgroups (*Polystichum* and *Davallia*) remained distinct from the rest of the taxa. The length of the 170 most parsimonious trees was 48, the CI was 0.83 and the RI was 0.65.

DISCUSSION

SIGNIFICANCE OF RELATIVELY HIGH *rbcl* SEQUENCE DIVERGENCE VALUES.—Compared to sequence divergence values of seed plants, those among *Polypodium* congeners (average value 1.87%) are apparently quite high. A survey of literature on phanerogam *rbcl* sequences failed to reveal studies comparing sequences within genera. Gadek and Quinn (1993), for example, reported that divergence values *between* genera of northern gymnosperms ranged from 0.6% to 2.6% and averaged 1.53%, and Conti et al. (1993) reported sequence divergence values as low as 0.9% and as high as 5.2% *between* genera in the Onagraceae.

High sequence divergence values for *Polypodium* help to substantiate data accumulated from other analyses (isozymes, restriction site analyses) indicating that ferns and seed plants may be fundamentally different at the level of interspecific genetic differentiation (Haufler, 1987; Soltis and Soltis, 1989; Yatskievych et al., 1988; Haufler et al., 1995a, b). All of the accumulated data indicate that fern species are on average more divergent from each other than are seed plant species. Wolf et al. (1994) reported sequence divergence values for other congeneric species that ranged from 0.2% in *Odontosoria* to 1.5% in *Doodia*. Because isozymic studies have demonstrated high genetic identity values among populations within species (Soltis and Soltis, 1990) and because little or no differences were seen when DNAs of different accessions of individual species were compared, it seems unlikely that there are cryptic species within each morphologically defined unit. Instead, perhaps these data indicate that fern speciation often requires that a large number of genetic changes accumulate before populations become isolated from each other at the species level. If such a model is accurate, speciation in ferns would be slower than that in seed plants.

The large number of nucleotide differences between *P. plesiosorum* and *P. rhodopleuron* (14) is particularly intriguing. As discussed in various floristic treatments (e.g., Mickel and Beitel, 1988), these two species are morphologically very similar; *P. rhodopleuron* is described as being less pubescent and having a greater accumulation of red pigment in the petioles and veins of its young leaves. These data suggest that additional molecular studies of infrageneric relationships in *Polypodium* are likely to be productive and informative.

THIRD POSITION "WOBBLE".—Among the taxa surveyed, nearly all of the phylogenetically informative sequence data were provided by variability at the third codon position. Because it is likely that third position variation has little or no effect on amino acid constitution, it is selectively "neutral." When such neutral characteristics emerge as potential species-specific markers, it seems reasonable to consider them phylogenetically reliable. Such appears to be the case in this study of the evolutionary relationships among *Polypodium* species.

THE TROPICAL SPECIES.—Based on a series of observations and the opinions of several tropical fern experts (see above), a set of Neotropical *Polypodium* species were hypothesized to represent the sister group to the temperate *P. vulgare* group. We excluded Paleotropical species from the analysis because Hennipman et al. (1990), Rödl-Linder, 1990, and Lellinger (1993) have argued that Paleotropical "polypodiums" are actually a distinct and separate genus, *Goniophlebium*. If it is reasonable to infer that congeners are more closely related than species from different genera, then taxonomic opinion also points to the Neotropics for relatives of the temperate *P. vulgare* complex. Among the Neotropical *Polypodium* species there is morphological evidence indicating that *P. plesiosorum* and related species are most closely related to the *P. vulgare* complex. Not only do the two groups share basic leaf shape features and similar venation patterns, but more cryptic aspects such as rhizome scale morphology and spore surface topographies also suggest a common history.

Previous restriction site analysis of the *P. vulgare* complex diploids had used *P. plesiosorum* as an outgroup species, and yet had demonstrated that this tropical species was so distinct from the temperate *P. vulgare* complex that it was difficult to develop accurate characterizations of some restriction site changes (Haufler et al., 1995a). The more conservative *rbcL* sequence data showed that the tropical polypodiums were reliably positioned as a close outgroup to the temperate species. Through all decay analyses and at a bootstrap confidence value of 92, members of the closely related Polypodiaceae genus *Pleopeltis* retained their sister-group relationship to the *Polypodium* species.

The sequence data also provided information on relationships among the three tropical *Polypodium* species. The species that appeared most closely allied morphologically (*P. plesiosorum* and *P. rhodopleuron*) also consistently emerged as sister species in this molecular analysis, whereas the morphologically distinctive *P. glaberulum* was more distant. This result suggested that it may be informative to obtain *rbcL* sequences of other tropical *Polypodium* species and that these data may help to resolve evolutionary relationships among these related, but ill-defined, species. In addition, it will be interesting to discover where *Goniophlebium* and scaly *Polypodium* species fall in an expanded molecular analysis of tropical Polypodiaceae.

THE *P. AMORPHUM* + *P. APPALACHIANUM* CLADE.—These two species have consistently emerged as closely allied sister taxa on the basis of morphology; they possess the synapomorphic soral feature called sporangiasters (Haufler and Windham, 1991). Studies of isozymic (Haufler et al., 1995b) and restriction site variation (Haufler et al., 1995a) also indicated that these species are nearest

TABLE 3. Genetic identities (Nei, 1972) based on isozyme analyses of *Polypodium scouleri* and other diploid *Polypodium* species. Figures in parentheses represent standard deviations.

	<i>P. scouleri</i>
<i>P. glycyrrhiza</i>	0.414 (0.408–0.435)
<i>P. appalachianum</i>	0.258 (0.216–0.498)
<i>P. rhodopleuron</i>	0.247 (0.240–0.273)
<i>P. pellucidum</i>	0.242 (0.141–0.303)
<i>P. plesiosorum</i>	0.212 (0.063–0.326)
<i>P. amorphum</i>	0.198 (0.160–0.253)
<i>P. glaberulum</i>	0.173 (0.172–0.174)
<i>P. macaronesicum</i>	0.184 (0.116–0.258)
<i>P. fauriei</i>	0.160 (0.157–0.168)
<i>P. australe</i>	0.018 (0.078–0.084)

neighbors. Although previous morphological and molecular studies demonstrated a close association between *P. appalachianum*, *P. amorphum*, and *P. sibiricum*, the *rbcL* sequence data are the first to link unambiguously this distinct clade to other temperate members of the *P. vulgare* complex. These sequence data, therefore, are consistent with the hypothesis that the *P. vulgare* complex is monophyletic.

THE *P. GYLCYRRHIZA* + *P. SCOULERI* + *P. FAURIEI* CLADE.—Although excluded from the *P. vulgare* complex in recent morphological studies, the association of *P. fauriei* with the complex was suggested earlier by Christensen (1928), and our sequence data support his assertions. By demonstrating a closer alliance than usually suspected between *P. fauriei* and other *P. vulgare* complex members, the *rbcL* data also provide a context for understanding the apparent involvement of *P. fauriei* in hybridization with *P. sibiricum* to originate the Japanese endemic *P. “okiense”* through allopolyploidy (Haufler et al., 1995a).

Because *P. scouleri* has such a distinctive set of morphological features, the sister taxon relationship suggested by the *rbcL* sequence data between this species and *P. glycyrrhiza* was unexpected. As an independent test of this surprising relationship, isozyme profiles were obtained for *P. scouleri* (following procedures discussed in Haufler et al., 1995b). Including species representative of the *P. vulgare* complex in the analysis, *P. scouleri* showed the highest genetic identity with *P. glycyrrhiza* (Table 3). In light of these molecular data and considering the ecology and biogeography of the species, it seems reasonable to hypothesize that *P. scouleri* could have originated through adaptation

to particularly stressful habitats. *Polypodium glycyrrhiza* has a relatively large range and is found primarily (but not exclusively) in forested, coastal regions from California north to Alaska and into Kamchatka. *Polypodium scolieri*, on the other hand, is rarely far from the ocean, confined to Pacific coastal habitats in the northern part of its range and volcanic substrates in warmer, drier, southern climates. It may be that the thick, leathery leaves characteristic of *P. scolieri* have evolved as an adaptational response to the stress of living in close contact with salt spray, wind, and/or volcanic substrates. Reproductive isolation may have evolved in concert with this physiological specialization and the additional morphological cues that we now use in recognizing *P. scolieri* (consistently netted venation, large sori) may have evolved later. In light of this hypothesis, molecular analysis of *P. californicum* may be particularly informative. There is a variant of this taxon, sometimes referred to as *P. californicum* var. *kaulfussii* D.C. Eaton, that has thicker-textured leaves, and inhabits extreme coastal localities. If after these additional taxa are included in the analysis *P. scolieri* remains more closely allied to *P. glycyrrhiza* than to *P. californicum*, it may be reasonable to propose parallel evolution of these adaptive leaf features in both cases.

THE *P. AUSTRALE* + *P. MACARONESICUM* CLADE.—The relationship between these two Old World diploids has been assumed for some time, because they have a similar set of gross-morphological features and both taxa have peculiar branched paraphyses associated with their sori. Although there has been some question about whether *P. macaronesicum* is a discrete taxon, the work of Roberts (1980) provided evidence of the morphological distinctness of the two species. The *rbcL* sequence data demonstrated that these two species differed by five base changes, which is equivalent to the amount of separation between the unambiguously distinct *P. amorphum* and *P. appalachianum*.

THE PERPLEXING *P. PELLUCIDUM*.—This strictly Hawaiian species contained a large number of autapomorphies (12 unambiguous nucleotide changes) and, based on these *rbcL* sequence data, the most reasonable indication of *P. pellucidum* relationships is displayed by the strict consensus tree (Fig. 1). However, depending on the kind of analysis that was performed, loose associations were obtained with various elements of the *P. vulgare* complex. For example, if PAUP displayed all compatible groupings when computing the consensus of the eight most parsimonious trees, *P. pellucidum* was associated with the *P. glycyrrhiza* clade with a 38% probability. In contrast, in a similar consensus tree after 100 bootstrap replicates, *P. pellucidum* was grouped with the *P. australe* clade at a 32% confidence level. Thus, although *P. pellucidum* appears solidly associated with other members of the *P. vulgare* complex, its sister group relationship remains unresolved.

In light of the hypothesis that *P. scolieri* and *P. glycyrrhiza* are sister species, it is interesting to note that in Hawaii *P. pellucidum* displays two distinct morphologies. Plants growing on volcanic substrates were accorded varietal status (*P. pellucidum* var. *volcanicum*) by Skottsberg (1941) because they had thick, imbricate pinnae and were quite different from the thin-leaved epi-

phytes growing in the rain forests. Ongoing studies of *P. pellucidum* (Li and Haufler, unpubl. data) indicate that there are no isozymic markers distinguishing the two presumed varieties and that when plants collected from volcanic substrates are grown in the greenhouse, some of the distinctive foliar features are lost.

THE *POLYPODIUM*/*PLEOPELTIS* BOUNDARY.—The new *rbcL* data support the recent controversial decision by Windham (1993) to emphasize indument features in clarifying the boundary between these two closely related genera. Prior to Windham's work, *Pl. thyssanolepis* had been considered part of *Polypodium*, yet the peculiar foliar, peltate scales appeared to ally it to *Pleopeltis*. The *rbcL* data grouped *Pl. thyssanolepis* unambiguously and consistently with *Pl. macrocarpa* var. *complanata* (Weatherby) Lellinger and not with *Polypodium*. Additional molecular studies of other *Polypodium* and *Pleopeltis* species may help to sort out remaining questions concerning this set of relationships and may yield a better understanding of phylogenetically significant morphological features among ferns in general.

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